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MATERIALS, TECHNIQUES, AND TESTING METHODS FOR THE SANITATION (BACTERIAL DECONTAMINATION) OF SMALL-SCALE WATER SUPPLIES IN THE FIELD USED IN GERMANY DURING AND AFTER THE WAR

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MATERIALS, TECHNIQUES, AND TESTING METHODS FOR THE SANITATION (BACTERIAL DECONTAMINATION) OF SMALL-SCALE WATER SUPPLIES IN THE FIELD USED IN GERMANY DURING AND AFTER THE WAR

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The purpose of visiting the above targets was to obtain detailed information about the nature, method, and specific bacteriological application of the Zsigmondy Membranfilter (membrane filter), which has been developed and is manufactured exclusively by the Membranfiltergesellschaft in Goettingen. The bacteriological applications have been and are still being studied and developed at several German Hygiene Institutes. Of these, the Hygiene Institute of the University of Goettingen (Prof. Dr. Buergers) and the Hygiene Institute connected with the University of Hamburg are particularly active or have collected detailed experiences during wartime.

The particular scientific and technological interest which was underlying the investigation of this target is based on the fact that the Zsigmondy Membranfilter technique represents a means of concentrating micro-organisms, in particular, bacteria, from even extremely dilute suspensions in aqueous liquids and especially water. To give an example: It is possible with the utilization of this technique to deposit within a short time (approximately 15 minutes) the entire bacterial flora suspended in 1 liter of water quantitatively on a membrane area of 40 cm^2 (6.2 sq. in.). The nature of the membranes permits subsequently the culturing of the micro-biological deposits on the membrane without transfer of the former in a number of ways, which leads to an identification in a time less than the usual culturing methods in liquid or gel-like nutrient media permit.

The practical application of this technique proved especially valuable for water tests, etc., in Germany during the war because of the simplicity of the methods and the lack of fragile laboratory equipment (bacteriological glassware and autoclaving facilities for it, etc.) and made it, for instance, possible to conduct the routine water tests in Hamburg after the laboratory facilities had been completely destroyed due to bombing.

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The membrane filter goes originally back to a development of Prof. Dr. Zsigmondy, Director of the Institute of Colloid Chemistry of the University of Goettingen, approximately thirty years ago. This development has subsequently gone through many variations and refinements in production, chiefly regarding lifetime and uniformity of the product. It was subsequently commercially produced by the Sartorius-Werke Aktiengesellschaft & Co., Goettingen, a well-known factory for scientific instruments, in particular, balances. Later a special division for the

manufacturing of this item and the equipment connected with it was established on the premises of the Sartorius-Werke AG. & Co., Weender Landstrasse 96/98, Goettingen, Germany. The operation and direction of the plant at the time of visit was in the hands of Mrs. Gertrud Keese and Prof. Dr. (Mrs.) A. Boling. The plant is in operation in order to supply numerous scientific and public health institutions.

The material of the Zsigmondy membranes consists of a thin layer of a complex structure of cellulose and cellulose esters, in particular in the latter case, of cellulose nitrate. The physical difference of these membranes from the usual filter papers or substances suitable for dialytic membranes is the practical absence of absorption of substance and retention only by physical screen action. This is possible due to the chemical neutrality of the material (practical absence of adsorptive and absorptive qualities) in one respect, and in the other, due to the great uniformity of the pores in the membrane. The size of the pores can be controlled to a large degree and down to unusually small orders of magnitude by the process of manufacturing. The average diameter of the smallest pores which can be manufactured cannot be determined directly; such membranes are capable of withholding quantitatively suspensions of Congo Red and proteins.

The control of the pore size is practicable in the range from $3 \cdot 10^{-4}$ cm. to the order of 10^{-6} cm. Consequently the application of these membranes is quite varied and not restricted to mechanical filtration. The pore sizes applied for bacterial retention of water-borne pathogens and E. coli are approximately 7×10^{-5} cm. (.7 micron).

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It was not the subject of the investigation to study the details of the manufacturing method except for establishing the fundamental principle of the procedure. This consists in the preparation of a dilution of one or several cellulose derivatives, in particular, cellulose nitrate, in a solvent to which a certain amount of water or other liquid, insoluble in the cellulose dilution, is added to form apparently a sort of emulsion in which the various components are distributed in a state of great uniformity in the size of minute droplets. The resulting liquid is then cast on plates and subsequently dried under carefully controlled conditions of temperature and humidity of the surrounding atmosphere. The resulting sheet is then cut and calibrated in order to determine the resulting pore size.

It was stated that the original composition, temperature of drying, and humidity during this process, determine the pore size within the range described.

The pore size is measured by the determination of a factor Z (Zeit = time) which is necessary to pass 100 cc. of distilled water at room temperature through a filter area of 100 cm^2 at a differential pressure of 1 at. (15 lbs.). The value of Z is, for the coarsest pore type filter, of the order of 5 sec., and for the finest, above 6000 sec.

If, as a practical example, the Coli concentration/index (or total bacterial count) of a sample of 100 cc. of water should be determined in the field with equipment involving the customary filter area of 12,5 cm^2, and if a membrane with the Z value (see above) of 15 sec. is used and the differential pressure is kept at ,5 at., the operation will require 4 minutes.

Two general types of membranes are manufactured, of which one type ("Membranfilter" and "Ultrafeinfilter") can only be used for the filtration of water and aqueous solutions, whereas the other type ("Zellafilter" and "Ultrazellafilter") can be used in contact with all common organic solvents. Both types of filters are resistant against:

H_2SO_4	up	to	20%
HCl	"	"	25%
KOH	"	"	3%
NaOH	"	"	3%
NH_4OH	"	"	8%

The filters of the former type are attacked by acetone, ether, chloroform, etc., but are resistant in temporary contact with dilute alcohol.

For the specific purpose of the present investigation, only filters of the former type with Z values of 10 - 30 sec. are interesting, and all references in the following concern this type.

A peculiar quality of these filter membranes, obviously quite desirable is the conical shape of the pores resulting from this method of manufacture, which is apparently due to the fact that in drying the rate of evaporation of the diluents differs between the upper and lower side of the cast film, which results in a difference of pore diameter between the upper and the lower side. The conical shape of a pore is particularly beneficial when the filter membrane is employed in such a manner that the side with the narrow pore diameter points upward, i.e., faces the unfiltered liquid, because the clogging of the pores is under these conditions practically absent. The side with the narrow pore size is matte, while the other is shiny.

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The equipment within which these membranes are used for bacteriological purposes varies with reference to the material from which it is manufactured (heat-resistant glass, porcelain, stainless steel), and with reference to the size of the filter area. For the bacteriological concentration from aqueous liquids, an effective area of the membrane of 12,5 cm^2 (approximately 2 sq. in.) is used. For the passage of larger quantities, for instance in the case of bacteriological air filtration, filter areas of 40 cm^2 (approximately 6 sq. in.) are probably more practicable.

The filter equipment does not differ in general design from the constructions used in this country for membrane filtration, except for

the fact that special precautions are taken for the somewhat delicate membrane to lie flat against the flanges and against the (ceramic) porous screen material which supports it. For the same reason, the precaution has to be taken during operation that the differential pressure across the membrane does not exceed .5 at. (7.4 lbs./ sq.in.).

The type of equipment used most commonly provides for a filtration by vacuum rather than by positive pressure. For the use of the membrane filter method in bacteriological investigations in the field, the whole equipment is in general made from stainless steel throughout in order to avoid fracture.

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The procedure for using filter membranes for the bacteriological investigation of water is, in principle, as follows:

The selected filter membrane is boiled two times for 15 minutes in water; in both cases the water is discarded. The purpose of this operation is the sterilization of the membrane and the removal of residual solvents from it. The membrane filter is then inserted into the filter apparatus under sterile precautions. The apparatus can previously be sterilized either by boiling or autoclaving, or simply by wetting with alcohol of every part and subsequent ignition.

When the filter membrane is inserted, it is important to notice that the matte side of the filter comes upward, because the growth of the bacteria on this side is more reliable (because of the conical pore shape).

For the filtration of water, it is important that the quantity of water is approximately adjusted to the bacterial content, so that after incubation the colonies can still easily be counted and appear as separate colonies. For instance: It may be advisable to filter (each time through a new membrane with the same F-value): 5, 50, 500 cc. of the water to be investigated. After the desired quantity of water has passed (if necessary, diluted with sterile water to avoid errors occurring from the use of too small quantities of water), the filter apparatus is taken apart; the filter membrane removed with sterile forceps, contacted with the particular nutrient agent required, and subsequently incubated.

As already mentioned initially, the value of these membrane filters rests not only on a relatively simple and speedy quantitative retention of an even minute number of micro-organisms from a large quantity of water upon a small area, but also upon the fact that with similarly very simple means, the membrane can be converted into a substrate which conveys the nutrients to the deposited bacteria and permits thus their culture in situ. This is possible because the pores of the membrane act as capillaries for a liquid or semi-liquid nutrient when such a membrane is brought with its lower side in contact with the medium. The liquid component of the nutrient is then delivered through the pores to the upper side of the membrane upon which the bacteria have been deposited by the previous filtration.

Although the method of culturing bacteria deposited on a membrane is in principle identical with the customary methods of culturing a bacterial inoculum upon the surface of an agar plate, the use of the membrane requires certain, though minor, variations in technique and in the composition of the nutrient. All these variations have the purpose of rendering the nutrient readily diffusible through the pores of the membrane; consequently, the agar content is reduced to one-half or one-third of the usual strength so that a semi-liquid medium results, from which the capillaries of the membranes remove easily the liquid component after contacting it. Dr. Mueller - Hamburg, has developed a technique which even disposes of the agar component in the nutrient completely, and renders the applicability of the method for field purposes particularly desirable (and was also very valuable during the war, due to the scarcity of agar in Germany).

The method consists in the impregnation of a wad of 3 - 6 sheets of standard laboratory filter paper with a liquid nutrient without any agar. These sheets are cut to fit closely into the bottom of a Petri dish, wherein they are sterilized. After filtration, the membrane is laid upon the impregnated paper in the dish. Since the capillary action of the pores in the membrane is larger than that of the filter paper, the former withdraw the nutrient from the latter.

The aforementioned desirability of using membranes of relatively small diameter (3 cm. or even less) is further increased by the possibility of placing a number (5) of such membranes within the same Petri dish; so that their surface is throughout in wetting contact with the nutrient without any part of one membrane overlapping another. This method effects a further saving of glassware, nutrient, and incubator space.

For the normal identification of E. coli collected from raw water samples, the Endo-type nutrient (Lactose, Peptone with Fuchsine) has been applied successfully to membrane cultures.

After incubation (37°C.) for 8 - 12 hours, the Coli colonies are easily recognized by the characteristic appearance of a metallic sheen. This identification renders — in contradistinction to Lactose fermentation — further confirmation on bile, etc., unnecessary.

Similarly, good results have been reported on nutrients containing Bromothymol blue as pH indicator.

The membrane filter method is also applicable to the specific identification of bacteria of the Salmonella type, especially of typhoid and para-typhoid. In these cases, the bismuth-sulfite nutrient (Wilson-Blair) is used, which is reduced to metallic Bi by B. typhi so that the colonies appear black with a metallic halo after incubation (37°C.) of 20 - 24 hours. The appearance of paratyphi B (Schottmuelleri) is almost identical except for larger colony diameters (3 - 5 mm. against 1 - 3 mm. for typhi). E. coli is not capable of reducing the nutrient to Bi to this degree; the colonies appear thus only as a light

brown without metallic sheen. Other micro-organisms are reported to have a sufficiently different appearance to permit easily the distinction of the pathogens and of E. coli.

Specific methods and technique for the successful application of these nutrients for membrane filters have been developed at the Hygiene Institute of the University of Hamburg during the war (G. Mueller). The work is described in detail in a monograph, entitled "Der Nachweis von Keimen der Typhus - Paratyphusgruppe im Wasser", by Dr. G. Mueller, Hamburg, available at the time of the investigation only as a manuscript.

When different specific media are used simultaneously for the same investigation, one membrane can be cut after filtration into several sectors, each of which can be contacted with a different nutrient in order to arrive at a multiple differentiation from one single sample, a method which is sometimes valuable under field conditions.

Finally, it may be mentioned that, after removal of the membrane from the nutrient at the end of the incubation period, the membrane (or its sectors) can be dried and kept as a permanent record with its stained colonies.

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Aside from bringing the bacterial deposits on the membrane into the range of visibility by culturing, it is also possible to use staining techniques in order to facilitate an immediate bacteriological inspection after the filtration since (after the displacement of the water in the pores with alcohol) the opaque membrane becomes almost completely transparent upon contact with immersion oil, Canada Balsam, or any other medium with a high index of refraction, which fact permits the use of the normal microscopic technique. It has, thus, been shown (Feldmann) that after the staining of the bacteria on the membrane, they can be directly observed with an immersion objective using the membrane as a carrier.

The staining of the bacteria is manipulated by permitting the dye to diffuse from below into the filter membrane where it reaches the bacteria deposited on the upper side. Subsequently, the excess of the dye solution is removed also by diffusion, by bringing the lower side of the membrane in contact with moist filter paper. The dye is then leached from the membrane, whereas it remains in the bacterial deposit. After subsequent drying, the membrane is penetrated with immersion oil and becomes transparent.

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The advantages of the membrane filter technique so far enumerated are chiefly of economical nature as far as media , equipment, and manpower are concerned. However, there has been impressed upon the investigator by the operators of this technique during the war years and subsequent times, that, in addition to this and the ease of operation, the method involves also definite bacteriological advantages over the

customary culturing methods on nutrient liquids and gels which appear more significant than the economic merits.

There exist numerous extended investigations which compare the Coli indices of the same raw water sample obtained by the customary methods (of serial dilution in liquid nutrients, plate cultures) and the membrane filter with the use of Endo nutrient. Especially when the raw water contained a varied flora, the counts of B.coli on the membrane were higher than those obtained by the customary methods. This is especially evident when the Coli indices were small and the total counts were high, in other words, when there exists a condition which suppresses the growth of Coli colonies due to interference by other micro-organisms (Saprophytes), or where the Coli organisms were of a low vitality already. In such cases, the growth of Coli was apparently most stimulated on the membrane and least on an agar plate surface, although the chemical composition of the nutrient was the same. The explanation offered for this phenomenon may be as follows:

The structure of the membrane permits diffusion only in one direction, i.e., from the nutrient to the surface of the membrane and the bacterial deposit thereon and vice versa, but almost none across the membrane, i.e., from one colony to another. At the surface of an agar plate, this is different and the chances for a diffusion of substance across the surface of the plate are as probable as the diffusion normal to the surface. If the flora contains a substantial quantity of organisms which inhibit the growth of Coli (apparently by means of the development of growth inhibitory bio-chemical agents), these inhibitors are obviously less likely to reach the Coli organisms on a membrane, in contra-distinction to the same situation on the surface of an agar plate. It stands thus to reason that the Coli count on the membrane will be larger than the Coli count obtained with the customary methods.

This peculiar physical property of the membranes, combined with the possibility of collecting bacteria from minute concentrations in water, explains why it was possible to identify and isolate also waterborne pathogens (typhoid and paratyphoid B.) in systematic investigations of various surface waters and the River Elbe near Hamburg, when the customary bacteriological investigations did not indicate their presence. In all these tests (Mueller), 500 cc. water samples were used, and the membranes were cultured on bismuth-sulfite membranes (see above).+

+ Of a series of 44 raw water samples taken at different tidal conditions, all proved negative for pathogens with the customary culture methods, while on the membrane: 5 tests (each for 500 cc.) proved positive for B.typhi, and/or Paratyphi B. The number of colonies so produced varied between 1 and 8.
Particularly significant for the capacity of this method for the differentiation of pathogens in the presence of a large general flora are the following results: 20 raw water samples were taken from different localities exposed to the city sewage, all of which had a total count of $2.5\text{-}3.5 \times 10^4$ and a Coli titer of 10^{-4}, i.e., a Coli factor (Coli count/total count) of 10 - 40%. In 10 of these 20 samples, it was possible to demonstrate pathogens: six showed B.typhi with an average of 2.5 colonies; three Paratyphi B., as 1, 3, and 20 colonies; one 2 Typhi and 3 Paratyphi colonies. These colonies were further identified with sub-culturing on a large variety of specific media and by serological agglutination.

There exists also an interesting difference in the time necessary for adequate incubation of membrane cultures of B.coli. Extended studies by Prof. Dr. H. Grossman and Prof. Dr. A. Beling, conducted during 1944 at the Institute of Hygiene at the University of Goettingen, investigated the dependence of the final Coli count on incubation interval. In these very careful studies, the water sample was filtered several times in succession through the same membrane in order to be certain that all bacteria had been retained on the membrane;+ artificial Coli inocula were used and the counts made after a total incubation time of 8, 10, 14, 16, and 24 hours, with the use of Endo-Fuchsine nutrient. All tests were conducted with an average Coli concentration in the water sample which resulted in 15-30 colonies on the total membrane area, that is, a concentration where the mutual inhibition of the colonies is not yet noticed. It is characteristic for 34 out of 35 readings in this test that the Coli count on the same membrane decreases with an increase of incubation time; consequently, the largest number of colonies are found after 8 hours, and the smallest after 24 hours. The increase of from 8 to 10 hours in general does not produce an apparent decrease, while the count obtained after 24 hours lies in general 20 - 30% below the reading obtained for the same plate at 8 - 10 hours. The reason for this apparent decrease is seen in the increase of the individual colony which, at a fairly close occupation of the membrane, produces a "fusion" of closely juxtaposed colonies into one complex which is subsequently recorded as one single colony, causing thus the apparent decrease in total count.

It is noteworthy that, apparently due to the uniform access to nutrient on the membrane, all individual Coli cells have developed already into colonies recognizable by the naked eye or through the magnifying glass after 8 - 10 hours, and are as such definitely identifiable as Coli. This fact would shorten the customary time necessary for incubation to a third, or even a sixth, which for certain purposes can be considered a definite advantage, especially since the time requirement for subsequent bio-chemical differentiation for "true" Coli is not necessary on account of the differential Fuchsine reaction.

It appears, however, doubtful that this advantage can be generalized to include all types of micro-organisms and media, since the studies with Typhi, etc., on Wilson-Blair nutrients indicate the necessity for an incubation period of 24 - 30 hours and thus no difference between the rate of colony development on the membrane and the agar surface.

+ This repeated filtration was an apparently unnecessary precaution since investigator was informed (Dr. Beling) that a pore diameter of 1 - 0.7 micron is definitely sufficient for the quantitative retention of E. coli during one passage of the water. There was also no indication that in any of the other tests described more than one passage had been used.

Investigator was informed that various successful attempts have been made for selective culturing of micro-organisms other than those mentioned above, e.g., yeasts and fungi, with the use of specific nutrients, but no detailed information about the methods developed or the results obtained could be made available, due to the limited time available to the investigator.

Samples were demonstrated which resulted from air filtration through membrane filters, which indicated the efficient and apparently quantitative retention of the organisms formerly suspended in micro-biological aerosols. In close analogy to the water filtration, a known quantity of air is sucked or pressed through a filter which has to be pre-wetted prior to the test. As already mentioned, larger filter areas (40 cm^2) are used in such experiments where it is then possible to culture fungi, yeasts, and bacteria on selective nutrients.

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The successful application of this technique during the war has caused a quite wide-spread interest in a general application through the various institutions concerned with the public health problems in postwar Germany, where the extreme lack of nutrients, glassware, autoclaves, trained personnel, etc., render these methods particularly useful.

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